Chapter 5

General Summary

A Heme Responsive Protein is Involved in the Regulation of CYP2B1/B2 Gene Transcription in Rat Liver

Primary interest in this laboratory centers on the regulation of eukaryotic gene expression The phenobarbitone (PB) inducible cytochrome P-450, CYP2B1/B2 gene in rat liver, is chosen as the model system CYP2B1/B2 gene belongs to a supergene family and is primarily regulated at transcriptional level CYP2B1 is 23 kb and CYP2B2 is 14 kb long and the two share a great degree of homology both in the coding and the upstream region except for a few minor differences Both the genes have 9 exons and 8 introns and are inducible by PB and in this investigation have been treated as a single unit

Studies in this laboratory have shown that sequences within -179 to +1 of the 5' upstream of the CYP2B1/B2 gene function as a minimal promoter, responsive to phenobarbitone A positive cis element, from -69 bp to -98 bp and a negative cis element from -127 bp to -160 bp have been identified within the minimal promoter It has been shown that the mechanism of induction of CYP2B1/B2 gene by PB involves fresh protein synthesis and phosphorylation of nuclear factors binding to the positive element

Earlier studies have also shown that heme, the prosthetic group of cytochrome P-450, is a positive modulator of transcription of this gene This was established on the basis that inhibitors of heme biosynthesis such as $CoCl_2$, 3-amino-1,2,4-triazole, thioacetamide and succinyl acetone inhibit PB-mediated induction of CYP2B1/B2 mRNA and its run-on transcription with isolated nuclei and this inhibition is counteracted by the administration of exogenous hemin

In the present study, the effect of $CoCl_2$ has been reexamined by using the sensitive method of RNase protection assay, since earlier results were based on filter hybridization and measurement of the radioactivity The nuclear and cytoplasmic RNAs were quantified using RNase protection assay with the I exon riboprobe The results clearly establish that $CoCl_2$ is a powerful inhibitor of CYP2B1/B2 mRNA induction by PB and this is counteracted atleast partially by exogenous hemin Run-on transcription experiments were carried out using I, VI and IX exon riboprobes and the results reveal that the inhibition caused by $CoCl_2$ administration is counteracted by exogenous heme Besides, in control nuclei the transcripts are not extended effectively to the IX exon but the transcripts reach the IX exon when treated with PB In $CoCl_2$ treatment once again the transcript levels are low when probed with IX exon Hemin treatment is able to counteract this situation These results showed that heme may play a role both in initiation as well as elongation of CYP2B1/B2 transcription

The effect of heme depletion was studied on the factors binding to the positive element from crude nuclear extracts Gel shift analysis with labelled Positive element and nuclear extracts revealed that $CoCl_2$ treatment inhibits complex formation and addition of heme *in vitro* and *in vivo* can overcome this effect The crude extract generates three complexes I, II and III, at optimal concentration of protein and these complexes are very faint in the case of heme depleted extracts When exogenous heme is administered the binding of all the complexes is enhanced The *in vitro* addition of heme is also effective at a concentration of 10^{-6} M PB treatment of the animal leads to an increase in the binding of the crude nuclear extract prepared from $CoCl_2$ treated rats to the labelled positive element This is also inhibited by $CoCl_2$ treatment and counteracted by exogenous hemin treatment

Attempts to identify the heme responsive protein(s) in the nuclear extracts have revealed that it is present in 0 5 M NaCl eluate from heparin agarose column used to fractionate the DNA binding proteins The 0 5 M heparin eluate binds the positive element in a heme responsive manner It enhances the formation of complex II Saline or PB treatments do not show this effect Protoporphyrin or iron cannot overcome the effect of $CoCl_2$ treatment

Further fractionation on positive element oligo affinity column yields a fraction which does not show heme responsive binding to the positive element However this fraction shows the ability to enhance transcription in a heme responsive manner This shows that the heme responsive factor is perhaps present in very low amounts, sufficient to enhance

transcription but not enough to manifest a detectable heme-responsive binding to the positive element in a gel shift assay

On this basis, the oligo affinity fraction was further purified on heme affinity column and this has lead to the isolation of a 65 kD protein that is responsive to heme The 65 kD protein as such binds very weakly to the positive element but augments binding of crude nuclear extract in a heme responsive manner Complex II shows maximum response This protein also enhances the complex formation with the negative element in presence of very small amounts of crude extract This enhancement is not as dramatic as that seen with the positive element The protein also stimulates transcription of a minigene construct containing -179 nt of the upstream of the CYP2B1/B2 gene in a cell-free transcription extract prepared from $CoCl_2$ treated rats in a heme responsive fashion

To test whether this protein makes a direct contact with the positive element, cross linking was carried out using the 0 5 M heparin eluate and the positive element The SDS-PAGE profile of the cross linking experiment shows a cross linked product which on subtraction of the molecular weight of the oligonucleotide shows a prominent band at 65 kD This shows that the 65 kD protein makes a direct contact with the positive element, but requires other protein factors to do so

All the binding assays indicate that this protein could be a heme binding protein To examine this, ^{59}Fe labelled heme was incubated with purified protein under appropriate conditions and the product was analyzed on SDS-PAGE, avoiding the boiling step and addition of β-mercapto ethanol The autoradiogram reveals a band at ~ 65 kD indicating that the protein binds heme

Studies in this laboratory have identified a 26 kD protein binding to the positive element and involved in the transcriptional regulation of the CYP2B1/B2 gene Earlier studies have implicated a 94 kD protein that might mediate protein-protein interaction The present studies reveal a 65 kD protein that regulates heme-responsive CYP2B1/B2 transcription involving the positive element It is visualized that all these interactions, that may involve both positive and negative effects, would eventually lead to interaction with a far upstream enhancer, accounting for the basal and PB-induced transcription of the gene Future study would involve an examination of these interactions